



## INTERNATIONAL APPLICATION PUBLISHED UNDER

WO 9605513A1

(51) International Patent Classification 6: <b>G01N 33/576, 33/50, 33/566</b>		A1	(11) International Publication Number: <b>WO 96/05513</b> (43) International Publication Date: 22 February 1996 (22.02.96)
(21) International Application Number: <b>PCT/IB95/00692</b>		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
(22) International Filing Date: 17 August 1995 (17.08.95)			
(30) Priority Data: <b>9416671.7 17 August 1994 (17.08.94) GB</b>			
(71) Applicant (for all designated States except US): BIOCINE S.P.A. [IT/IT]; Via Fiorentina, 1, I-53100 Siena (IT).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventor; and			
(73) Inventor/Applicant (for US only): ABRIGNANI, Sergio [IT/IT]; Piazza Roma, 13, I-53035 Monteriggioni (IT).			
(74) Agent: HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).			

(54) Title: ASSAY TO DETECT HCV RECEPTOR BINDING

## (57) Abstract

Identification of HCV receptor target cells using HCV receptor-binding ligands and cell separation by flow cyt fluorimetry. HCV receptor target cells are employed to conduct assays for HCV receptor-binding ligands in order to identify potential HCV vaccine candidates. HCV receptor target cells are used to measure antibody neutralisation to monitor vaccine development, as a diagnostic of HCV infection and to develop neutralising antibodies for passive immunisation.

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## ASSAY TO DETECT HCV RECEPTOR BINDING.

Field of the Invention

5 The present invention relates to an assay to measure binding of hepatitis C virus (HCV) receptor binding ligand, such as HCV proteins, to a target cell receptor. The assay may be used to evaluate vaccine candidates and to identify and measure HCV neutralising antibodies both for research  
10 purposes and clinical applications where diagnosing the presence of neutralising antibodies may have a prognostic value in clinical management.

15 The invention also relates to identifying and characterising the receptor for HCV which will facilitate the identification and screening of antivirals that interfere with receptor interaction.

Brief Description of the Prior Art

20 Hepatitis C virus (HCV - previously known as Non-A Non-B hepatitis - NANBV) has become a major health problem worldwide, since 1-2 % of the population is chronically infected with HCV (1). Infection is mostly asymptomatic and  
25 resolution occurs in a minority of cases, since 80 to 100 % of the infected individuals become lifelong carriers (2) and chronic hepatitis develops in the majority of these cases (3).

30 HCV is a positive sense RNA virus of about 10000 nucleotides with a single open reading frame encoding for a polyprotein of about 3000 amino acids. Although the structure of the virus has been elucidated by recombinant DNA techniques (17,18), the virus itself has not been isolated and the  
35 functions of the various viral proteins produced by proteolysis of the polyprotein have only been inferred by analogy with other similar viruses of similar genomic organisation.

The viral proteins are all available in recombinant form, expressed in a variety of cells and cell types, including yeast, bacteria, insect and mammalian cells (5,10)

5

Two proteins, named E1 and E2 (corresponding to amino acids 192-383 and 384-750 respectively) have been suggested to be external proteins of the viral envelope (5) which are responsible for the binding of virus to target cells.

10

We have devised a method for identifying cells carrying a putative HCV receptor and assay techniques for detecting and quantifying the binding of HCV receptor-binding ligands to the receptor. The technique has a wide range of 15 applications and utilities.

A first step in designing an HCV vaccine is the identification of the components involved in protective immunity. At present, little is known on the role the immune 20 response plays in the course of HCV infection.

The identification of HCV receptor target cells facilitates the characterisation of the HCV receptor itself and provides an important component in the development of assays for 25 binding of HCV receptor-binding ligands to HCV receptor target cells. Such assays may be used in the diagnosis of neutralising antibodies in individuals, the rapid screening of antiviral compounds which interfere with receptor binding and the development of vaccines.

30

In a passive immunization study in chimpanzees, HCV infection has been prevented after *in vitro* neutralization with plasma of a chronically infected patient (6). However, the assessment of protective antibody responses to HCV has 35 been hampered by the absence of a neutralization assay *in vitro*. Since HCV does not grow efficiently in cell cultures, attempts have been made (7,8) to set up neutralization tests that estimated HCV binding to target cells. However, the

available tests are based on the detection of bound virus by PCR, with obvious shortcomings such as the difficulties in quantitating neutralizing antibodies and problems in obtaining accurate reproduction of RT-PCR testing.

5

The invention also relates to a quantitative test (named Neutralisation of binding or N.O.B.) to estimate HCV neutralizing antibodies which is based on the cytofluorimetric assessment of sera that neutralize the 10 binding of HCV envelope proteins to human cells.

#### Summary of the Invention

According to a first aspect of the present invention, there 15 is provided an assay for measuring the binding of an HCV receptor-binding ligand to an HCV receptor target cell comprising the step of measuring the binding of an HCV receptor-binding ligand or a competitive binding analogue thereof to a HCV receptor target cell.

20

Preferably, binding of the HCV receptor-binding ligand or the HCV receptor-binding ligand analogue is detected by labelling bound species and employing a detection system capable of distinguishing between free HCV receptor target 25 cells and bound HCV receptor target cells.

Preferably, the detection system is flow cytometry, more preferably flow cytofluorimetry. In this case, bound species carry a fluorescent label and physical cell 30 separation occurs between labelled and unlabelled cells.

The first aspect of the invention provides a sensitive and fast assay for the ability of a possible HCV receptor-binding ligand to bind to an HCV receptor target cell and 35 facilitates ready screening of possible HCV receptor-binding ligands. Such possible HCV receptor-binding ligands may have utility as antiviral agents or as possible vaccine or assay reagent candidates.

The assay may be a direct binding assay, measuring directly the binding of a HCV receptor-binding ligand to an HCV receptor target cell or may be a competitive assay in which 5 HCV receptor-binding ligand to be measured in a sample competes for binding with an HCV receptor-binding ligand analogue and the amount of HCV receptor-binding ligand analogue is measured.

10 In a direct binding assay, the assay steps comprise

- i) admixing HCV receptor target cells and a sample to be tested for the presence of HCV receptor-binding ligand to permit binding of any HCV receptor-binding ligand 15 with the HCV receptor target cells
- ii) removing unbound HCV receptor-binding ligand
- iii) admixing with a detectable antibody capable of binding 20 to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and
- iv) detecting the amount of labelled HCV receptor target 25 cells.

The detectable antibody may be directly labelled or may be detectable by adding a labelled ligand such as an antibody, suitably one specific for the fixed region of the detectable 30 antibody.

The label may be any label capable of directly or indirectly indicating the presence of the label. Preferably, however, the label is a fluorochrome suitable for use in flow 35 cytometry. Suitable such labels include fluorescein-isothiocyanate (FITC), phycoeriphosphine (PE) and Texas Red.

The labelled ligand might be any other ligand capable of

binding specifically to the detectable antibody. For example, the detectable antibody might itself have covalently-linked biotin and the labelled ligand might be streptavidin.

5

The detectable antibody may be, for example, a human, rabbit or mouse immunoglobulin such as IgG and the labelled antibody may be a labelled anti-human, anti-rabbit or anti-mouse antibody.

10

The detectable antibody or the second labelled antibody may be a polyclonal or monoclonal antibody. In either case the antibody may be a binding fragment of an antibody, such as a F(ab') fragment. The monoclonal antibody may be produced 15 by a cell fusion technique or by a recombinant DNA technique such as humanisation or CDR grafting.

In the described example, the detectable antibody is a polyclonal antibody (e.g. rabbit serum) raised against the 20 HCV receptor-binding ligand in an immunised animal host and the second, labelled, antibody is an anti-animal host (e.g. anti-rabbit IgG) antibody labelled with FITC. Preferably however the detectable antibody is a monoclonal antibody.

25 A control amount of antibody of the same type as the detectable antibody may be added in a parallel experiment as a control. The control amount may be for example a pre-immune serum.

30 In an indirect binding assay, the assay steps comprise

1) admixing HCV receptor target cells, a sample to be tested for the presence of HCV receptor-binding ligand and a limiting amount of an HCV receptor-binding ligand analogue to permit competition for binding to the HCV receptor target cells,

35

ii) removing unbound HCV receptor-binding ligand

- iii) admixing with a detectable antibody capable of binding to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and
- iv) detecting the amount of HCV receptor target cells bound to the HCV receptor-binding ligand analogue.

10 In either case, the amount of labelled HCV receptor target cells may be detected by providing a fluorescent label and performing cell separation using flow cytometry.

According to a second aspect of the invention, there is

15 provided an assay for measuring neutralisation of an HCV receptor binding ligand arising from the binding of a neutralising antibody to an HCV receptor binding ligand (referred to above as an NOB assay) comprising the steps of:

- i) admixing HCV receptor target cells, a HCV receptor-binding ligand and a sample to be tested for the presence of an HCV neutralising antibody
- ii) removing unbound HCV receptor-binding ligand,
- iii) admixing with a detectable antibody capable of binding to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and
- iv) detecting the amount of HCV receptor target cells bound to the HCV receptor-binding ligand.

In this assay, HCV receptor target cells and HCV neutralising antibodies compete for binding to the HCV receptor-binding ligand.

The second aspect of the invention provides a sensitive and

fast assay for identifying antibodies capable of impairing the binding of HCV to HCV receptors. Such antibodies are likely to neutralise the virus and to be one of the effective responses of the immune system arising from a 5 successful immunisation. The level of neutralising antibodies is therefore indicative of the degree of success of an immunisation protocol and neutralising antibodies may themselves be useful as the active principle in pharmaceutical preparations for passive immunisation.

10

The assay of the second aspect of the invention may also provide a sensitive assay for detecting HCV antibodies in a sample as a method of diagnosis of existing or previous HCV infection. Preferably therefore the sample is a sample from 15 a patient and the invention provides a method for diagnosing an existing or past infection with HCV comprising conducting the assay of the second aspect of the invention on a sample removed from patient.

20 We have discovered in chimpanzees immunised with E1/E2 produced in HeLa cells that there is a perfect correlation between the presence of neutralising antibodies and protection from a subsequent challenge with homologous HCV. Since the animals with the highest titre remained completely 25 free of virus, we assume that the antibodies are neutralising antibodies induced by the vaccination. Effective neutralisation titres are still present one year after the third boost. Low titres are followed by mild infection which unlike controls was resolved by vaccination.

30

The capability for measuring neutralising antibodies thus permits diagnosis of past or present HCV infection and has a prognostic value in the treatment of infected patients.

35 The present assay permits the characterisation of neutralising antibodies and may be used directly as an assay on patient samples or may be used to validate and test reagents for the development of sensitive assays suitable

for use in the routine clinical environment. Such assays would include for example enzyme-labelled assays, particularly ELISA.

- 5 The assay of the second aspect of the invention may also be useful in identifying a panel of antibodies capable of binding to the HCV receptor enabling mapping of the receptor on HCV receptor target cells.
- 10 Finally, the assay of the second aspect of the invention may be useful in determining cross-reactivity between antibodies to HCV external proteins from different HCV genotypes.

15 The reagents used in the second aspect of the invention may be as described in the first aspect of the invention and the protocols applied *mutatis mutandis*.

According to a third aspect of the present invention, there is provided a method for identifying HCV receptor target 20 cells in a sample of cells comprising the steps of:

- i) admixing the sample of cells and an HCV receptor-binding ligand to permit binding of any HCV receptor-binding ligand with any HCV receptor target cells in 25 the sample
- ii) removing unbound HCV receptor-binding ligand,
- iii) admixing with a detectable antibody capable of binding 30 to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and
- iv) detecting the amount of labelled HCV receptor target 35 cells.

The reagents used may be as described in the first aspect of the invention and the protocols applied *mutatis mutandis*.

The method of the third aspect of the invention provides a rapid screening technique for cells carrying an HCV receptor. The method may therefore be used to produce the

5 HCV receptor target cell of the first and second aspects of the invention or to produce HCV receptor target cell populations for subsequent analysis and in particular characterisation of the nature and form of the HCV receptor for the purposes of further refining and improving available

10 HCV receptor-binding ligands.

The invention also encompasses products produced or identified using the assays and methods of the invention, including HCV receptor-binding ligands identified using the

15 assays of the invention and vaccine compositions including same. Any development programme involving an assay or method of the present invention falls within the scope of protection sought by this application.

20 As used herein, the term "HCV receptor target cell" refers to a cell or cell population capable of binding at least one HCV protein. Suitably, the HCV receptor target cell contains at least one HCV protein receptor.

25 As used herein, the term "HCV receptor-binding ligand" refers to any ligand capable of binding to an HCV receptor. The HCV receptor-binding ligand preferably comprises one or more HCV polypeptides and/or one or more fragments of an HCV polypeptide capable of binding to an HCV receptor. The HCV

30 receptor-binding ligand may be a polypeptide unrelated to HCV yet capable of binding to an HCV receptor. Suitably the HCV receptor-binding ligand is a recombinant HCV protein or a fragment thereof. Such HCV receptor-binding ligands are disclosed in European patent applications EP-A-0318216 and

35 EP-A-0388232. Most preferred are HCV external proteins E1 and E2 or functional equivalents and fragments thereof which retain the ability to bind to an HCV receptor target cell.

Alternatively, the HCV receptor-binding ligand may be non-polypeptide chemical compound capable of binding an HCV receptor. The non-polypeptide chemical compound may be a chemical analogue of a receptor-binding polypeptide, 5 retaining the receptor-binding epitope.

As used herein, the term "HCV receptor-binding ligand analogue" refers to an analogue of an HCV receptor-binding ligand which is capable of cross-reacting with the HCV 10 receptor-binding ligand for binding to the HCV receptor target cell but is distinguishable from the HCV receptor-binding ligand using the assay detection system. Suitably therefore, the HCV receptor-binding ligand analogue is labelled preferably with a fluorescent label either directly 15 or by the further binding of a labelled ligand such as an antibody.

As used herein the term "polypeptide" refers to a sequence of two or more amino acids comprising at least one peptide 20 bond. The amino acids in the sequence may be naturally occurring amino acids or may be synthetic analogues or wholly synthetic amino acids in any mixture or ratio. The term "polypeptide" encompasses generically proteins and modified polypeptides such as naturally or chemically 25 modified polypeptides, including glycoproteins.

As used herein, the term "HCV neutralising antibody" refers to an antibody capable of reducing the interaction between an HCV receptor-binding ligand and a HCV receptor target 30 cell. Preferably the HCV receptor-binding ligand is a native HCV protein. Preferably the HCV neutralising antibody completely prevents binding of a native HCV protein to HCV receptor target cells. HCV neutralising antibodies may be monoclonal antibodies (produced, for example by the 35 Köhlr and Milstein technique of cell fusion or by recombinant DNA techniques such as humanisation and CDR grafting) but are preferably polyclonal antibodies, most preferably antibodies produced by the immune system of an

immunised host.

Brief Description of the Drawings

5 Figure 1 is a schematic diagram illustrating the first aspect of the invention in which HCV receptor-binding ligands bind to receptors on HCV receptor target cells and are measured by first binding rabbit anti-HCV antibody and then by binding a labelled anti-rabbit IgG-FITC F(ab')  
10 fragment prior to cell separation by FACScan analysis (See page 14).

Figure 2 is a representation of the results of flow cytometry experiment describing the differential binding of  
15 HCV recombinant envelopes expressed in various systems to human cells. Molt-4 cells incubated with medium alone are shown as hatched curves and the open curves represent cells incubated with 5 µg/ml of the indicated HCV recombinant envelopes (See page 16).

20 Figure 3 shows the binding of E2 expressed in CHO cells to human cells (See page 16).

Figure 4 is a schematic diagram illustrating the second  
25 aspect of the invention in which a neutralising antibody prevents an HCV receptor-binding ligand binding to the receptor on a HCV receptor target cell (see page 17).

Figure 5 shows neutralisation of the binding of E2 to target  
30 cells by antibodies to the hypervariable region 1 of HCV E2 (see page 19)

Figure 6 shows the neutralisation of E2 binding by HCV target cells by vaccinated chimpanzee sera, demonstrating  
35 the correlation between protection and the presence of neutralising antibodies (see page 19).

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, cytofluorimetry and molecular biology, which are within the 5 skill of the art. Such techniques are explained fully in the literature (19).

The skilled person will understand and be familiar with the general methods and techniques of assay design and practice. 10 The invention is described herein in sufficient detail for the skilled person to understand and repeat the experiments disclosed.

It will be understood by the skilled person that alterations 15 of the conditions may be necessary to optimise the assay for given HCV receptor-binding ligands, HCV receptor target cells and antibodies.

Standard abbreviations for virus and proteins are used in 20 this specification. All publications, patents and patent applications cited herein are incorporated by reference. Envelope 1 (E1) and Envelope 2 (E2) of HCV refer to the proteins, and fragments thereof, the nucleotide sequence of which are published (17,18). The nucleotides of the E1 and 25 E2 genes and of the encoded proteins vary in different HCV isolates. Therefore, the E1 and E2 for any HCV isolates are identified because included in the amino acid sequences 192-383 and 384-750 respectively.

30 E1 and E2 have been produced by recombinant DNA techniques using different expression systems (5,10).

#### General Materials and Methods

35 Cells. The human T-c 11 lymphoma cell line, Molt-4 was obtained from ATCC (Rockville, MD). Cells were expanded with RPMI 1640 (Gibco Laboratories, Grand Island, NY) medium supplemented with 2 mM L-glutamine, 1 % nonessential amino

acids, 1mM sodium pyruvate, penicillin (100 units / ml), streptomycin (100 µg / ml), and 10 % (vol / vol) Foetal calf serum (FCS, Gibco).

5 Recombinant envelope proteins The glycoproteins E1/E2<sub>100-745</sub> were expressed in HeLa or CHO cells, extracted and purified as described (5,9). E2<sub>384-715</sub> was expressed and secreted from recombinant CHO cells as described for the truncated E2<sub>384</sub> (5). For purification of CHO/E2, CHO cells conditioned media 10 was concentrated 15 fold by ultrafiltration, followed by a further 10 fold volume reduction by ammonium sulphate precipitation at 75% saturation, and redissolution into 25 mM Tris-Cl, 1 mM EDTA, pH 7.5; the monoclonal antibody 5E5/H7 (specific for CHO/E2) was purified and coupled onto 15 CNBr-activated Sepharose. The antibody column was equilibrated in 25 mM Tris-Cl, 0.15 M NaCl, pH 7.5. The ammonium sulphate precipitated E2 was dissolved in 25 mM Tris-Cl, 1 mM EDTA, pH 7.5, and loaded onto the column. The column was washed with PBS plus 1-M NaCl, and then eluted 20 with 3-4 column volumes of Actisep (Sterogene Inc., Arcadia, CA). All of the yellow-coloured Actisep containing fractions were pooled, concentrated in a stirred cell ultrafilter and diafiltered into PBS buffer. E2<sub>384-715</sub> was expressed and secreted from recombinant Baculovirus (BV) infected cells as 25 described (10). For purification of BV/E2, conditioned medium from insect cells was loaded onto a column of GNA lectin agarose (Vector Laboratories, Burlingame, CA). The column was then washed with PBS plus 0.9 M NaCl, and eluted with 1 M methyl D-mannoside in PBS plus 0.9 M NaCl. The 30 eluate was dialysed against 20 mM potassium phosphate, pH 6, at 4°C. The precipitate, containing mostly contaminants, was removed by centrifugation, and the supernatant loaded onto a column of S-Sepharos Fast Flow (Pharmacia, Uppsala, Sweden) equilibrated in 20 mM potassium phosphate, pH 6. 35 The E2 protein was loaded with a gradient to 0.25 M NaCl in 20 mM potassium Phosphate, pH 6. For expression and secretion from yeast of E2 384-715, we used the *Saccharomyces cerevisiae* strain S150-2B and the secretion

vector YEpsec1 (11). E2 is secreted as a core glycosylated peptide of 55 KDa. Yeast/E2 was purified by affinity chromatography using a lectin column and the same procedure used for purification of BV/E2 (10). After purification, all 5 the HCV envelope proteins were > 80% pure. ELISA for all antigens were performed according to published procedures (10).

Sera and monoclonal antibodies (mAbs). Rabbit polyclonal 10 antiserum specific for all the envelope proteins described above and sera from chimpanzees that have been immunized with HeLa E1/E2 or with a combination of Yeast/E1<sub>1-1111</sub> and BV/E2<sub>404-461</sub> (9) were obtained. The monoclonal antibody 291 (IgG1) was obtained from mice immunized with CHO/E2 and 15 screened for the ability to recognise E2 bound to target cells. A synthetic peptide consisting of HCV-1 amino acids 384-414 (E2 hypervariable region 1, HVR1) was coupled through the amino terminal residue to Diphtheria toxoid and used to immunize mice. The mAbs resulting from the fusion 20 were screened by Elisa with overlapping biotinylated 8mer peptides from amino acid 288 to 487 on streptavidin coated plates. An IgG1 mAb (1G2A7) was isolated which recognise in ELISA the epitope 384-414.

25 Example 1 - Binding Assay

A schematic representation of a binding experiment is shown in Figure 1 which shows the separation achieved by flow cytometric analysis.

30 An experiment was performed with the aim of measuring the ability of HCV protein to bind to various cell types which should have the putative HCV receptor.

35 Experimental Protocol

Indirect immunofluorescence experiments were performed to assess the ability of HCV envelope proteins to bind to Molt-

4 cells, which is a human T-cell lymphoma that has been reported to allow a low level of HCV replication *in vitro* (13).

5 Cells ( $10^5$ /well) Molt-4 were pelleted in 96 U-bottom microplates by centrifugation at  $200 \times g$  for 5 min at  $4^{\circ}\text{C}$ .  
10  $20\mu\text{l}$  of HCV proteins diluted in PBS at different concentrations (from  $10 \mu\text{g/ml}$  to  $0.001 \mu\text{g/ml}$ ) were mixed with the pellet of Molt-4 cells and incubated at  $4^{\circ}\text{C}$  for 1 hr. Non-bound HCV proteins were removed by two centrifugations in PBS at  $200 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Cells were subsequently incubated for 30 min at  $4^{\circ}\text{C}$  with various dilutions of sera from humans, chimpanzees, or rabbits that had been either infected with HCV or immunized with HCV  
15 recombinant proteins; where possible, the corresponding pre-immune sera were used as control. The cells were washed twice in PBS and incubated for 30 minutes with the appropriate dilution of fluorescein-isothiocyanate-conjugated antiserum (either to human IgG or rabbit IgG).

20 Cells were subsequently washed in PBS at  $4^{\circ}\text{C}$ , resuspended in  $100 \mu\text{l}$  PBS and cell-bound fluorescence was analysed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). By using a dot plot display of forward and side scatter, the machine is gated to include viable single cells and to exclude cell debris and clumps of cells. A total of 25 5000 events is collected and analyses of the data is done by using the Lysis II software program from Becton Dickinson. This program produce histograms of each cell sample and 30 calculates the mean channel fluorescence of the cell population, which directly relates to the surface density of 35 fluorescently labelled HCV proteins bound to the cells. Mean fluorescence values (mean channel number) of cells incubated with or without HCV proteins and with immune or preimmune sera were compared. The threshold for positivity is set for each experiment by flow cytometric analysis of cells with HCV proteins bound which have been incubated with antisera to HCV proteins and the FITC labelled second antibody.

The experiment described above shows that binding of E2 to target cells is measurable and has high affinity.

5 An experiment was conducted to compare the ability of different HCV proteins expressed in various systems to bind various cell types possessing an HCV receptor.

Cells were incubated with HCV recombinant envelopes (E1/E2 10 or E2), expressed either in yeast, insect cells or mammalian cells (HeLa or CHO), and subsequently incubated with polyclonal sera from rabbits that have been immunized with the corresponding recombinant proteins. After incubation with FITC-conjugated antiserum to rabbit IgG, the binding of 15 HCV proteins was indirectly detected by flow cytometry as cell-bound fluorescence.

The representative experiments in Figure 2 show that recombinant E1/E2 or E2 expressed in mammalian cells, but 20 not in yeast, can bind human cells, whereas E2 expressed in insect cells has a low, but detectable binding. Identical data were also obtained using as target cells hepatocarcinoma cell lines or freshly purified human B cells.

25 After incubation of the target MOLT-4 cells with increasing concentrations of E1/E2 or E2, it was found (Figure 3A) that the binding of E2 expressed in mammalian cells plateaued at a concentration of 10  $\mu$ g/ ml.

30 Since this binding is saturable, the affinity of recombinant E2 for its putative receptor could be estimated using the double reciprocal plot method previously described for the calculation of the affinity of hapten-antibody interaction 35 (14). In Figure 3B, the estimated affinity is expressed as  $K_d$  and it is equal to the reciprocal of the free E2 concentration at which half the concentration of E2 is bound to its putative receptor. In the y-axis, the neat mean

fluorescence intensity values for each concentration of E2 was calculated by subtracting the mean fluorescence obtained with rabbit anti-E2 serum and FITC-goat anti-rabbit in the absence of E2 from that obtained in the presence of E2. The 5 neat mean fluorescence intensity (y-axis) and the E2 concentration (x-axis) were plotted as reciprocal values.

The  $K_d$  of E2 for target cells is about  $10^{-6}$ M leading to the conclusion that E2 is probably the protein responsible for 10 the specific binding of the E1/E2 complexes to target cells.

#### Example 2 - Neutralisation Assay

A schematic representation of a neutralisation assay 15 according to the invention is shown in Figure 4.

#### Experimental Protocol

Cells ( $10^5$ /well) from were pelleted in 96 U-bottom 20 microplates by centrifugation at  $200 \times g$  for 5 min at 4°C.  $20\mu\text{l}$  of  $\text{CHO}/\text{E2}_{15}$  ( $0.5 \mu\text{g}/\text{ml}$  PBS) were mixed with various dilutions of sera from humans, chimpanzees or rabbits that are either infected with HCV or have been immunized with HCV recombinant proteins. After incubation at 4°C for 1 hr, 25 Molt-4 cells were added and incubated for 1 hr at 4°C. Non-bound HCV proteins and antibodies were removed by two centrifugations in PBS at  $200 \times g$  for 5 min at 4°C. Cells were subsequently incubated for 30 min at 4°C with 1/100 dilution of sera, from the same species of the neutralizing 30 serum, from animals that have been immunized with HCV-envelope recombinant proteins or the corresponding pre-immune sera as control. Revealing the binding with antibodies from the same species of the neutralizing serum is critical, since non-neutralizing anti-E2 antibodies could 35 cover E2 after it is bound to target cells and could therefore interfere with assessment of neutralization if the binding were revealed with an anti-E2 serum from a different species. The cells were washed twice in PBS and incubated

for 30 min with the appropriate dilutions of FITC-conjugated antiserum to IgG. Cell-bound fluorescence is analysed as described for the binding assay above.

5 This technique is very helpful to measure cross-neutralisation of antibodies to HCV envelope proteins from various HCV genotypes. For instance, antibodies raised against envelope proteins from HCV genotype 1A can be assessed for their ability to neutralise binding of Envelope 10 proteins from HCV genotype 1b, 2, 3 etc.

#### **Antibodies to the E2 Hypervariable Region Neutralise Binding**

An experiment was conducted to test whether the binding 15 measured according to Example 1 is neutralisable with antibody to E2. Rabbit polyclonal antisera specific for the recombinant E2 expressed in CHO cells, was assessed for ability to neutralize binding of E2.

20 E2 (at concentration of 0.5 µg/ml, i. e., the Kd) was mixed with serial dilutions of the rabbit antisera. The E2-antibody mixture was then incubated with target cells, and the binding of E2 was subsequently detected. It was shown that sera from rabbits immunized with E2 expressed in 25 mammalian cells can neutralize binding of E2 to target cells.

Since for other viruses, epitopes able to induce neutralizing antibodies have been located in regions showing 30 a high degree of variability, further experiments investigated whether a mAb against the HCV-E2 hypervariable region 1(aa 384-414) neutralized binding of E2.

CHO/E2 was preincubated with the indicated concentrations 35 of the purified mAb (1G2A7) specific for HCV-E2 hypervariable region 1 (HVR1). The antibody-E2 mixture was then incubated with Molt-4 cells and the binding was revealed using monoclonal antibody 291 which recognises E2

bound to target cells. Mean fluorescence intensity (MFI) values in the absence of neutralizing mAb (positive control), in the absence of E2 (negative control) and in the presence of E2-antibody complexes (experimental values) were 5 measured and specific neutralization was determined according to the equation: Specific neutralization =  $x 100$  [( positive control MFI - experimental MFI) / (positive control MFI - negative control MFI)]. The results are presented in Figure 5.

10

Figure 5 shows that the HVR1 specific mAb can neutralize, though not completely binding of E2 demonstrating that binding of E2 is at least in part mediated by hypervariable regions.

15

**Antibodies that neutralize binding of HCV envelope correlate with protection from infection**

It has been shown that vaccination with recombinant envelope 20 proteins expressed in mammalian cells (HeLa), but not in yeast or insect cells, could protect chimpanzees from primary infection by an homologous HCV isolate (9).

To investigate whether the binding, and subsequent 25 neutralization, of E2 were relevant to the binding of HCV to target cells, the neutralizing titres present in the sera of chimpanzees vaccinated and protected from subsequent challenge were compared with the sera from chimpanzees immunized but susceptible to HCV challenge.

30

The results are depicted in Figure 6 in which the results for each chimp were as follows:

Chimp:	559
35 Vaccine:	HELA E1/E2
Outcom :	Prot ct d
Anti-E1/E2 ELISA Titre:	1/37900
Anti-E2 HV ELISA Titre:	1/49

50% Neutralisation Titre: 1/3500

Chimp:	357
Vaccine:	HELA E1/E2
5 Outcome:	Protected
Anti-E1/E2 ELISA Titre:	1/25900
Anti-E2 HV ELISA Titre:	1/30
50% Neutralisation Titre:	1/2500
10 Chimp:	534
Vaccine:	HELA E1/E2
Outcome:	Protected
Anti-E1/E2 ELISA Titre:	1/22300
Anti-E2 HV ELISA Titre:	1/64
15 50% Neutralisation Titre:	1/600
Chimp:	653
Vaccine:	HELA E1/E2
Outcome:	Protected
20 Anti-E1/E2 ELISA Titre:	1/8700
Anti-E2 HV ELISA Titre:	ND
50% Neutralisation Titre:	1/1500
Chimp:	470
25 Vaccine:	HELA E1/E2
Outcome:	Infected but resolved
Anti-E1/E2 ELISA Titre:	1/5300
Anti-E2 HV ELISA Titre:	1/95
50% Neutralisation Titre:	1/250
30 Chimp:	WS181
Vaccine:	HELA E1/E2
Outcom :	Infected but resolved
Anti-E1/E2 ELISA Titre:	1/2600

Anti-E2 HV ELISA Titre: 1/ND  
50% Neutralisation Titre: 1/300

5 Chimp: 590  
Vaccine: Yeast E1/BV E2  
Outcome: Infected  
Anti-E1/E2 ELISA Titre: 1/4300  
Anti-E2 HV ELISA Titre: ND  
10 50% Neutralisation Titre: 1/0

Chimp: 635  
Vaccine: Yeast E1/BV E2  
Outcome: Infected  
15 Anti-E1/E2 ELISA Titre: 1/2800  
Anti-E2 HV ELISA Titre: 1/42  
50% Neutralisation Titre: 1/0

Figure 6 and the above data show that all chimpanzees that  
20 had NOB neutralizing titres of at least 1/600 were protected  
from infection, chimpanzees with NOB titres of about 1/300  
developed a mild infection and resolved, whereas chimpanzees  
with no NOB antibodies were not protected (9).

25 Serial dilutions of sera from chimpanzees vaccinated with  
recombinant envelope proteins (9) were tested for their  
ability to neutralize binding of E2. In each square is  
indicated the envelope proteins used as vaccine, the ELISA titres  
30 against HeLa E1/E2 and the peptide corresponding to the E2-  
HVR1, and the 50% neutralization titres calculated as in  
Figure 5.

Figure 6 also shows that ELISA titres to E2-hypervariable region 1 were comparable in protected versus non-protected chimpanzees demonstrating that E2-binding neutralizing antibodies correlate with protection from infection, and 5 that neutralization induced by vaccination does not depend on antibodies to the HVR1.

In parallel, sera from human infected with a given HCV genotype can be tested for ability to neutralise binding of 10 envelope proteins from HCV genotypes different from the infecting genotype.

It will be understood that the invention is described above by way of example and modifications within the scope and 15 spirit of the invention may be made without the need for undue experiment or the exercise of inventive ingenuity.

References

1. Van der Poel, C. L. et al (1994) *Lancet* 344, 1475-1479.
- 5 2. Alter, M.J., et al (1992) *N. Engl. J. Med.* 327 1899-1905.
3. Alter, M.J., et al (1989) in *Current Perspectives in Hepatology*, eds Seef, L.B. et al (Plenum, New York) 83-97.
- 10 4. Choo, Q. -L. et al (1991) *Proc. Natl. Acad. Sci. USA* 88, 2451-2455.
5. Spaete, R. R. et al (1992) *Virology* 188, 819-830.
6. Farci, P. et al (1994) *Proc. Natl. Acad. Sci. USA* 91, 7792- 7796.
- 15 7. Shimizu, Y. K. et al (1994) *J. Virol.* 68, 1494- 1500.
8. Zibert, A. et al (1995) *Virol.* 208, 653- 661.
9. Choo, Q. -L. et al (1994) *Proc. Natl. Acad. Sci. USA* 91, 1294-1298.
10. Chien, D. Y. et al (1992) *Proc. Natl. Acad. Sci. USA* 89, 10011-10015.
- 20 11. Baldari, C. et al (1987) *EMBO J.* 6, 229-234.
12. Lau, J. Y. N. et al (1995) *J. Infect. Dis.* 171, 281-289.
13. Shimizu, Y. K. et al (1992) *Proc. Natl. Acad. Sci. USA* 89, 5477-5481.
- 25 14. Celada, F. et al (1973) *Immunochemistry* 10 797-804.
15. Weiner, A. J. et al (1992) *Proc. Natl. Acad. Sci. USA* 89, 3468-3472.
16. Botarelli, P. et al (1993) *Gastroenterology* 104 580-587
- 30 17. European patent application EP-A-0318216
18. European patent application EP-A-0388232
19. Virology, Eds Dulbecco R. et al Harper & Row Philadelphia 1980.

CLAIMS

1. An assay for measuring the binding of an HCV receptor-binding ligand to a target cell comprising the step of  
5 measuring the binding of an HCV receptor-binding ligand or a competitive binding analogue thereof to a HCV receptor target cell.

2. An assay according to claim 1 wherein the assay steps  
10 comprise:

- i) admixing HCV receptor target cells and a sample to be tested for the presence of HCV receptor-binding ligand to permit binding of any HCV receptor-binding ligand  
15 with the HCV receptor target cells
- ii) removing unbound HCV receptor-binding ligand
- iii) admixing with a detectable antibody capable of binding  
20 to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and
- iv) detecting the amount of labelled HCV receptor target  
25 cells.

3. An assay according to claim 1 wherein the assay steps comprise:

- 30 i) admixing HCV receptor target cells, a sample to be tested for the presence of HCV receptor-binding ligand and a limiting amount of an HCV receptor-binding ligand analogue to permit competition for binding to the HCV receptor target cells,

ii) removing unbound HCV receptor-binding ligand

5 iii) admixing with a detectable antibody capable of binding to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and

10 iv) detecting the amount of HCV receptor target cells bound to the HCV receptor-binding ligand analogue.

4. An assay for measuring neutralisation of an HCV receptor binding ligand arising from the binding of a neutralising antibody to an HCV receptor binding ligand  
15 comprising the steps of:

i) admixing HCV receptor target cells, a HCV receptor-binding ligand and a sample to be tested for the presence of an HCV neutralising antibody

20 ii) removing unbound HCV receptor-binding ligand,

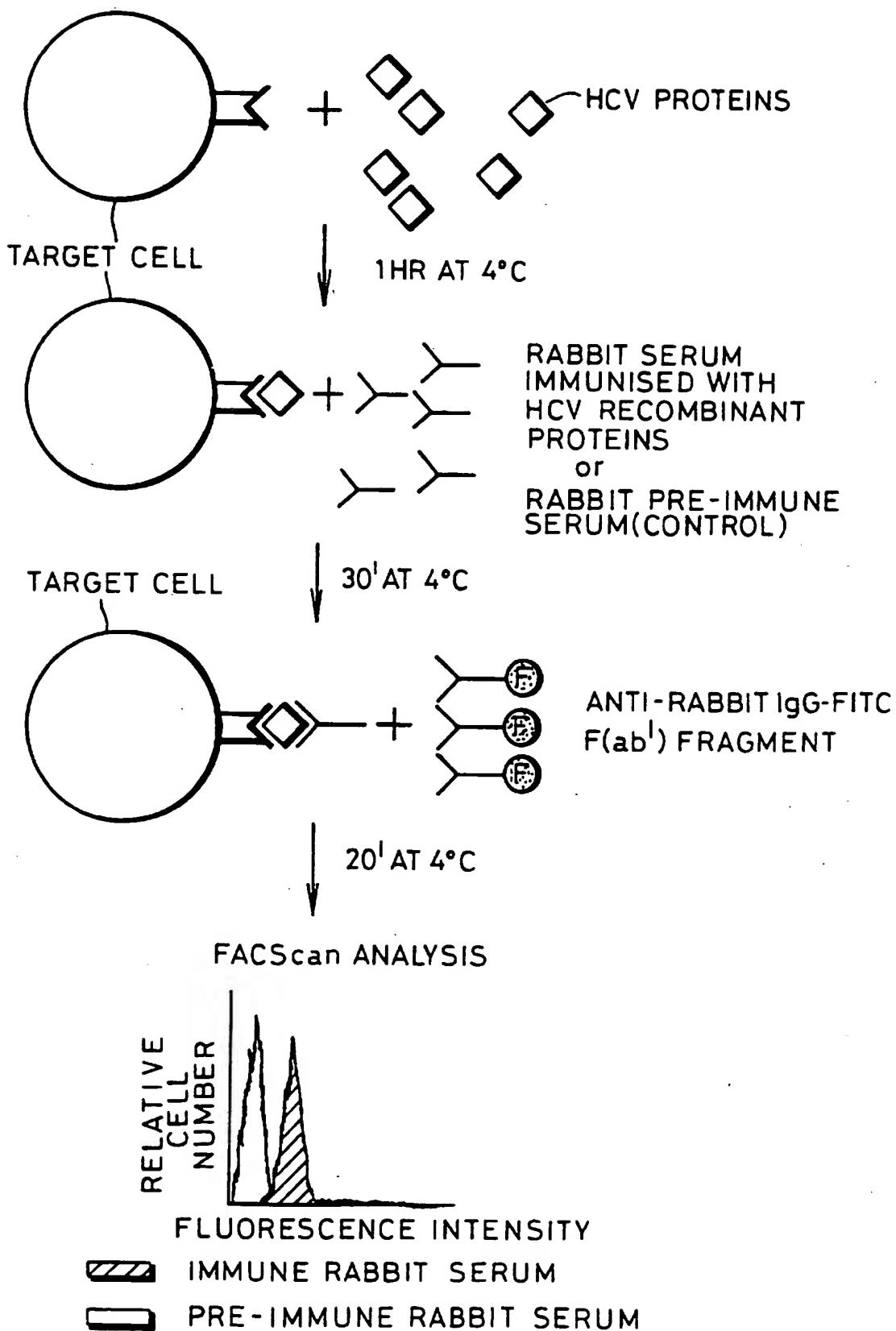
25 iii) admixing with a detectable antibody capable of binding to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and

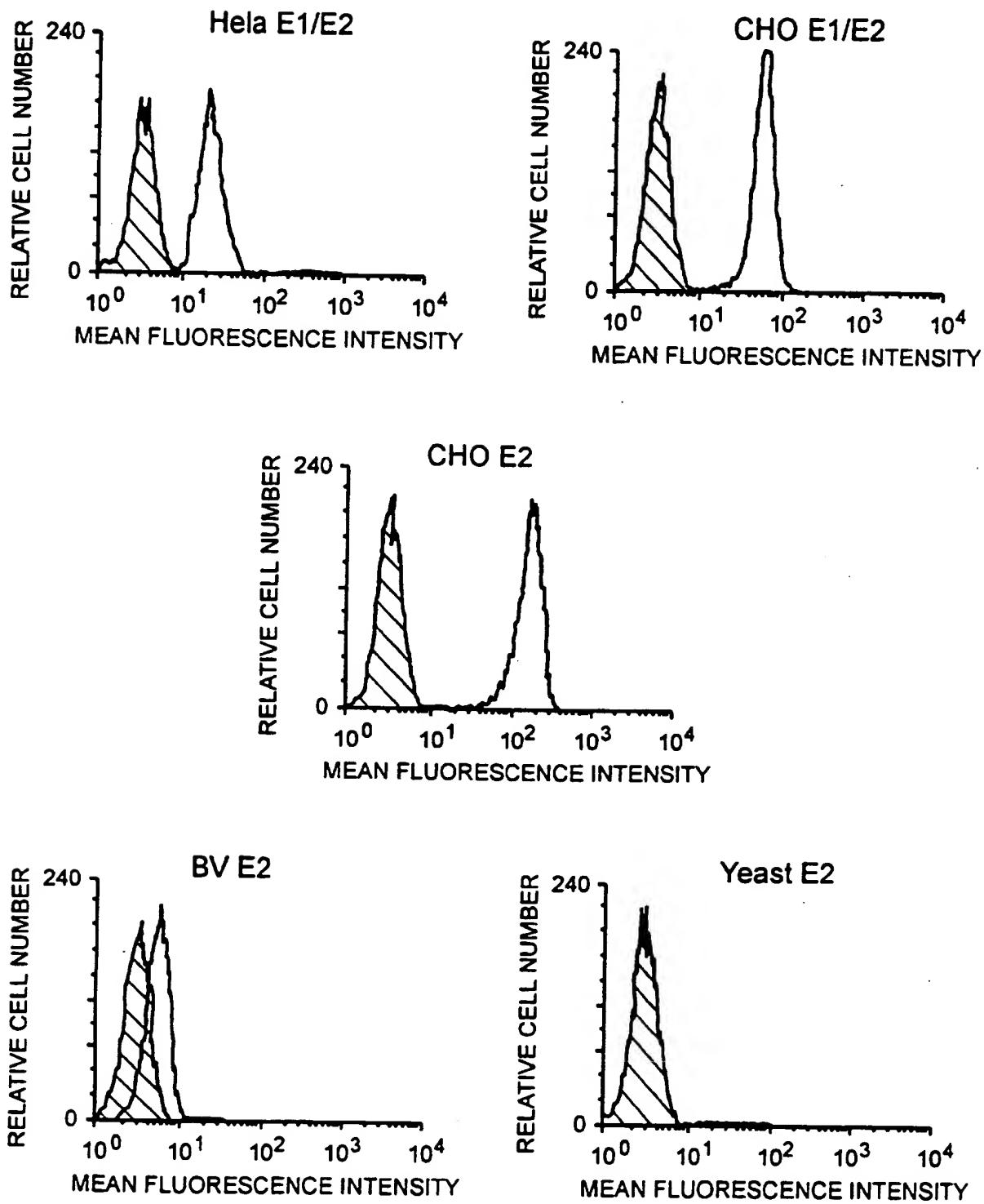
iv) detecting the amount of HCV receptor target cells bound to the HCV receptor-binding ligand.

30 5. A method for identifying HCV receptor target cells in a sample of cells comprising the steps of:

- i) admixing the sample of cells and an HCV receptor-binding ligand to permit binding of any HCV receptor-binding ligand with any HCV receptor target cells in the sample
- 5 ii) removing unbound HCV receptor-binding ligand
- 10 iii) admixing with a detectable antibody capable of binding to the HCV receptor-binding ligand to label those HCV receptor target cells which have bound HCV receptor-binding ligand, and
- 15 iv) detecting the amount of labelled HCV receptor target cells.

6. An assay or method according to any one of the preceding claims wherein the amount of HCV receptor target cells bound to the HCV receptor-binding ligand is detected by flow cytometry.

**FIG.1**

**FIG.2**

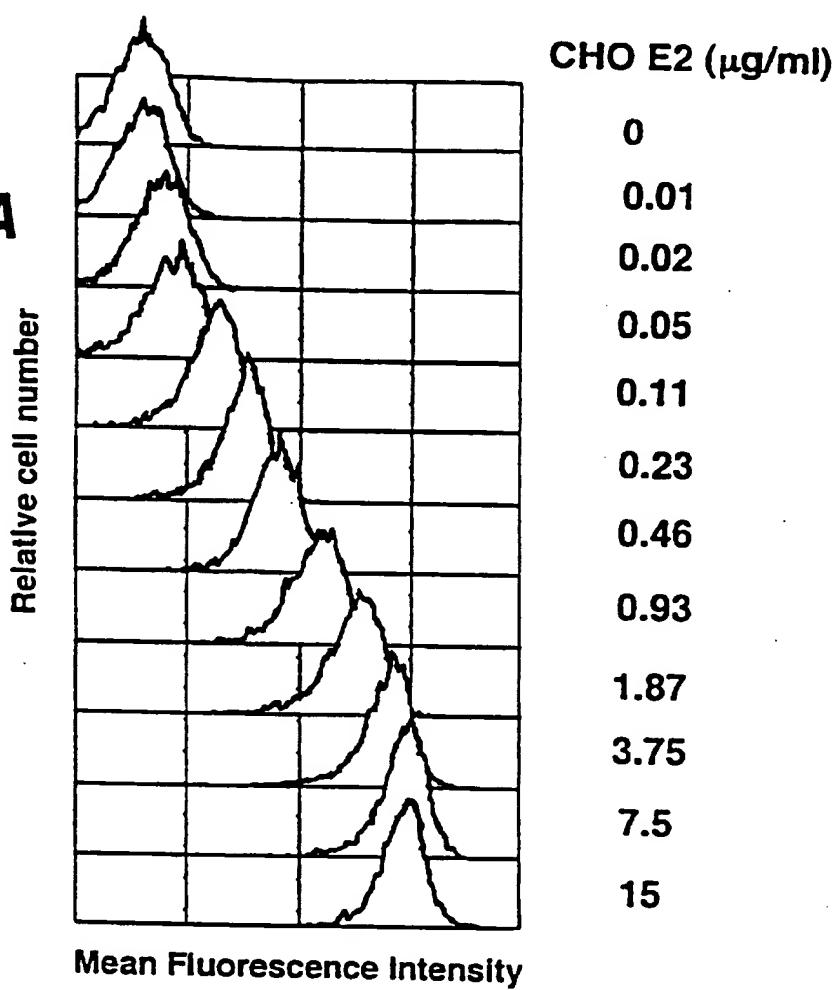
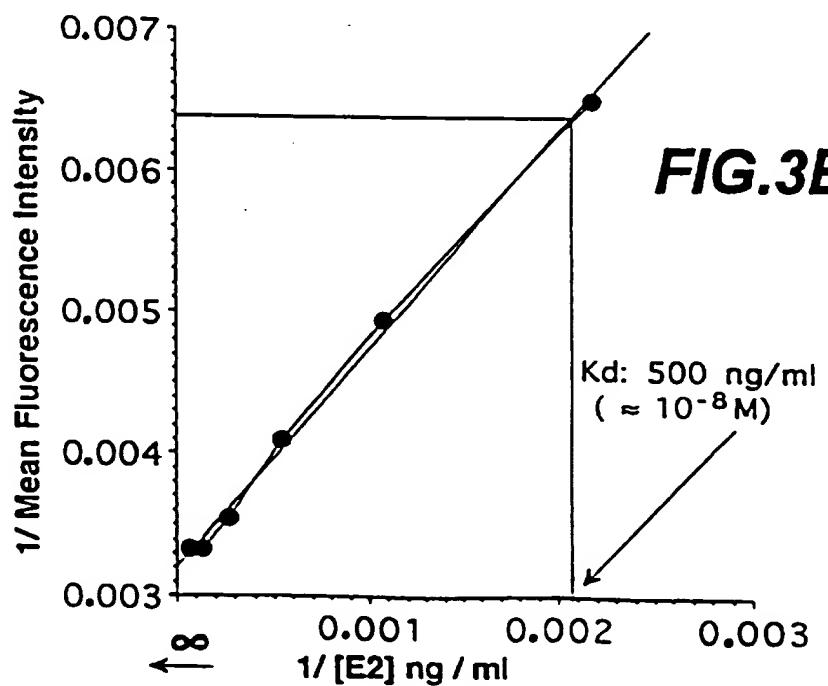
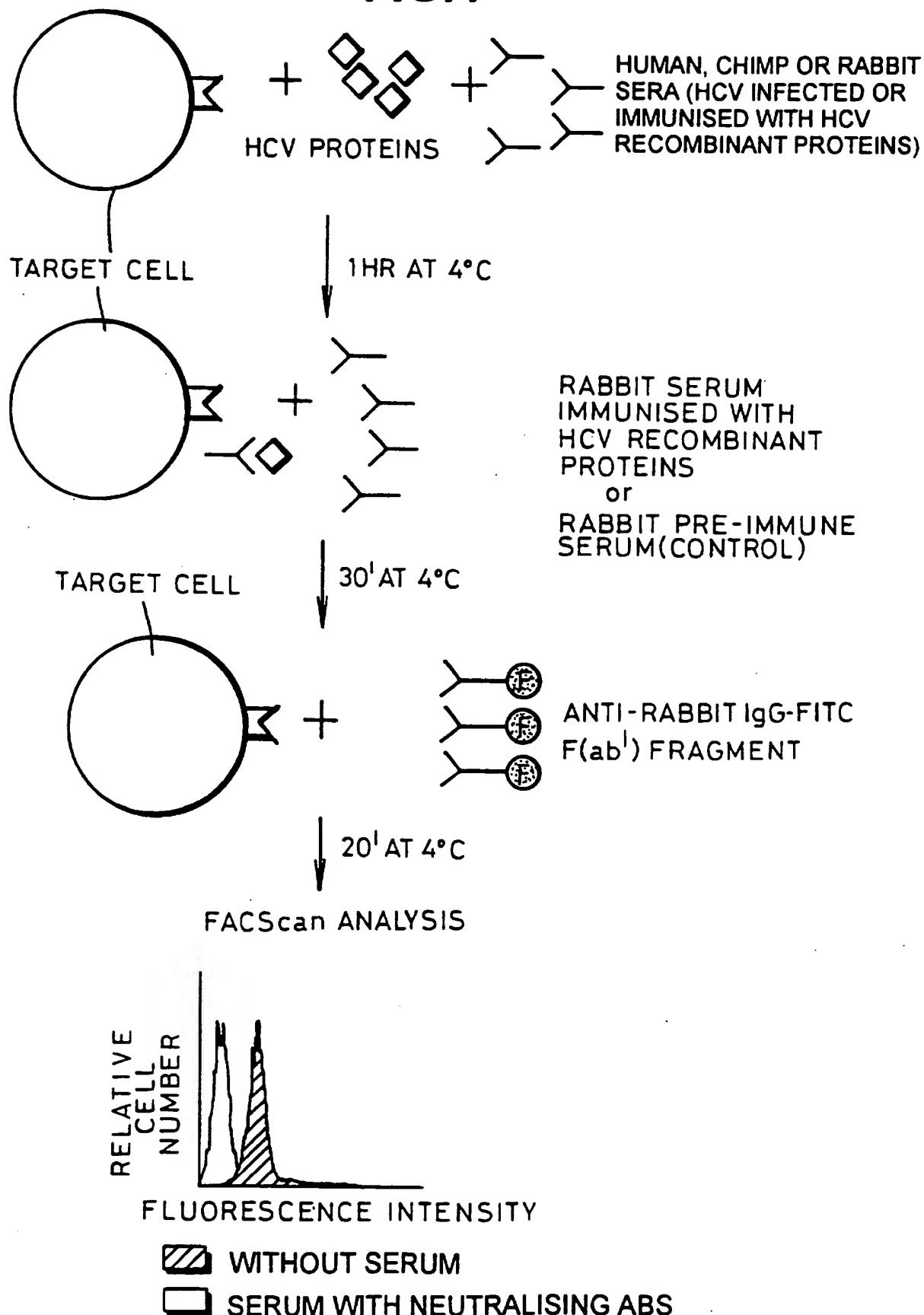
**FIG.3A****FIG.3B**

FIG.4



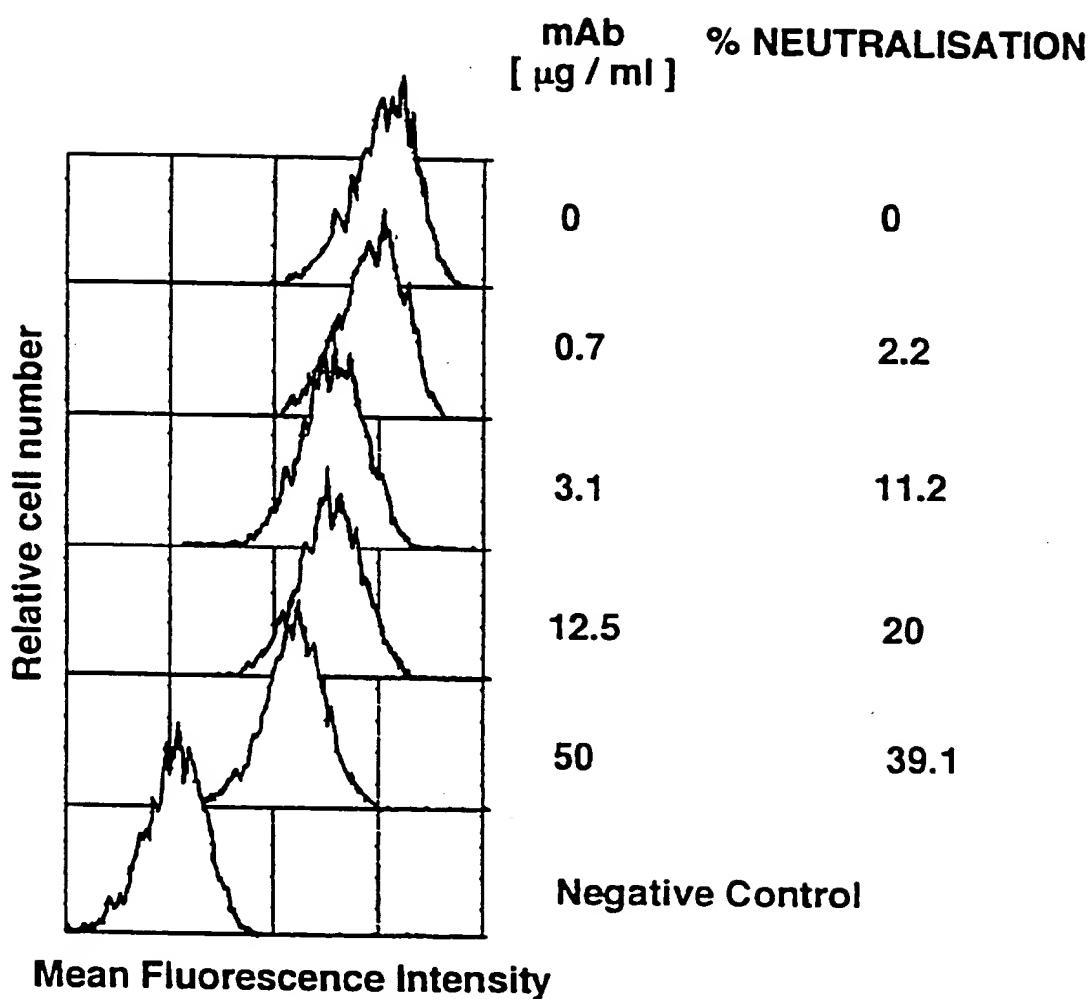
**FIG.5**

FIG. 6

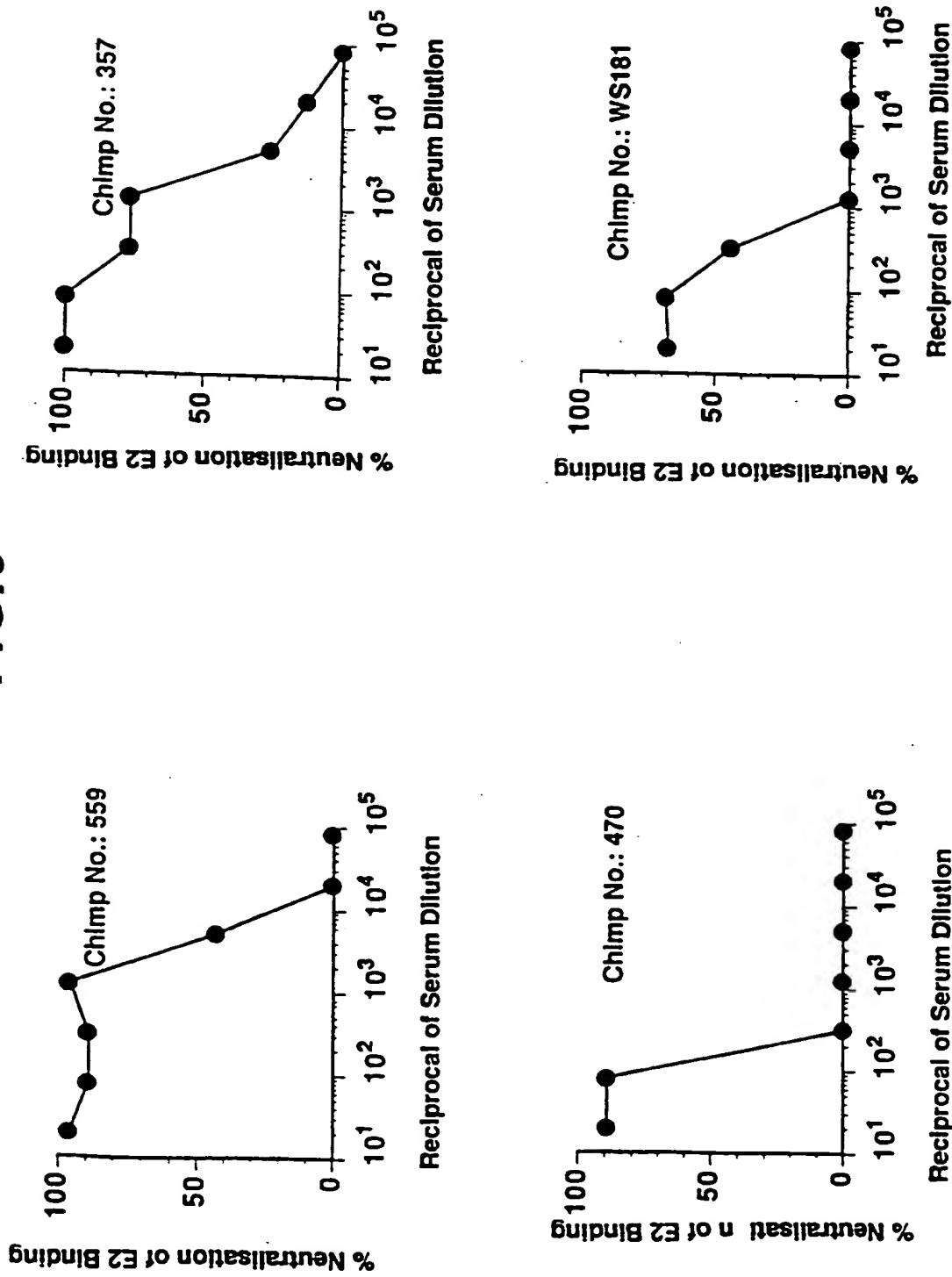
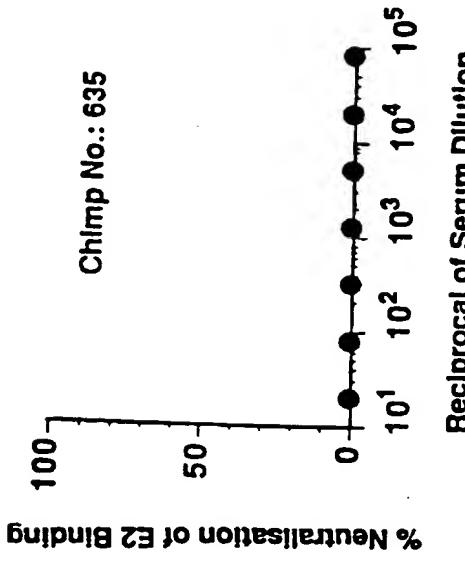
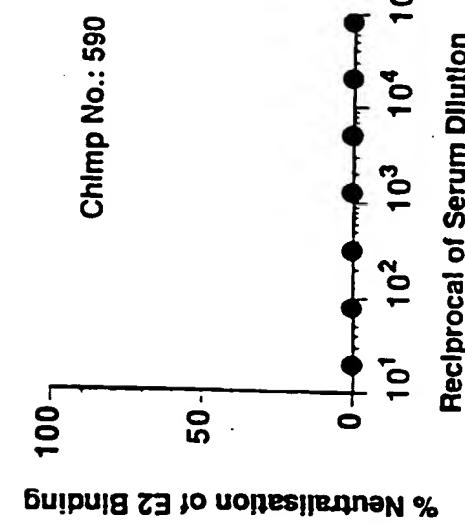
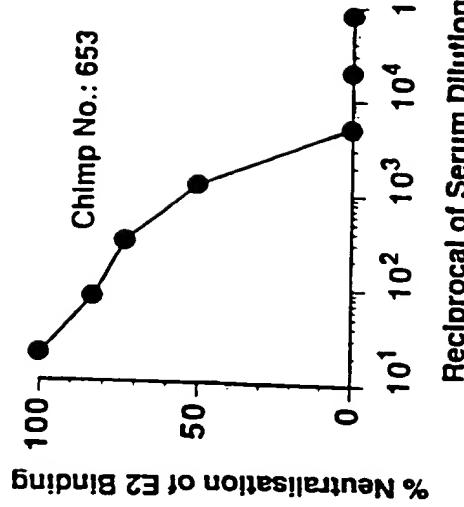
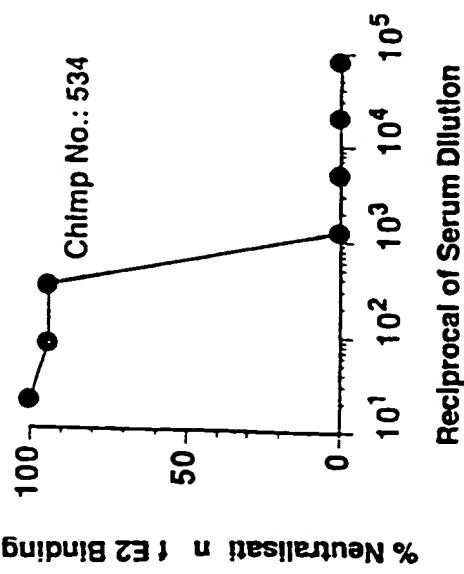


FIG. 6 (contd.)



## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB 95/00692A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/576 G01N33/50 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF EXPERIMENTAL MEDICINE, vol. 178, 1 July 1993 NEW YORK NY USA, pages 17-25, M.A. MINUTELLO ET AL. 'Compartmentalization of T lymphocytes to the site of disease: intrahepatic CD4+ T cells specific for the protein NS4 of hepatitis C virus in patients with chronic hepatitis C.' see figure 1 see the whole document -----	1
A		2-6

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*'A' document defining the general state of the art which is not considered to be of particular relevance
- \*'E' earlier document but published on or after the international filing date
- \*'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*'O' document referring to an oral disclosure, use, exhibition or other means
- \*'P' document published prior to the international filing date but later than the priority date claimed

- \*'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*'&' document member of the same patent family

2

Date of the actual completion of the international search  30 November 1995	Date of mailing of the international search report  08.12.1995
Name and mailing address of the ISA  European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl. Fax (+ 31-70) 340-3016	Authorized officer  Van Bohemen, C